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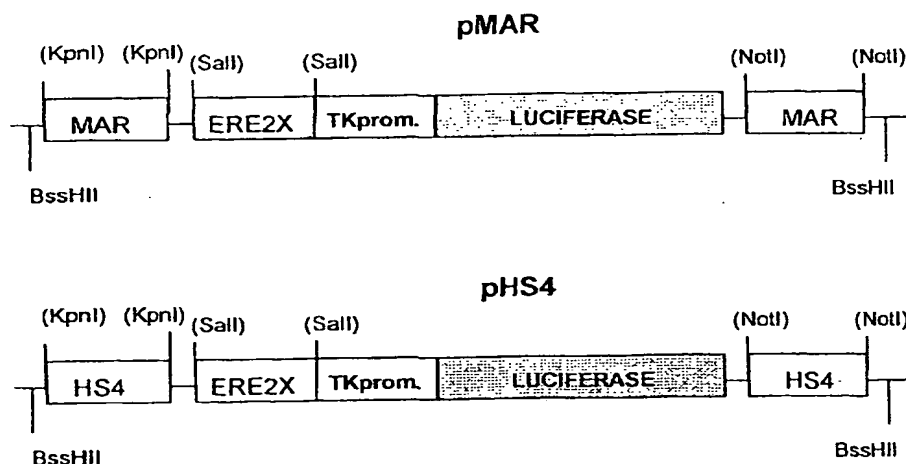
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(54) Title: **TRANSGENIC MOUSE FOR SCREENING AND FOR STUDIES OF THE PHARMACODYNAMICS AND PHARMACOKINETICS OF LIGANDS ACTING ON THE OESTROGEN RECEPTOR AND ITS INTRACELLULAR RECEPTORS, AND METHOD FOR THE PREPARATION THEREOF**



Final constructs - Ligation of the MAR and HS4 sequences
in the flanking regions of the pERE2XTKluc construct.

(57) Abstract: The object of the invention is a method for the production of a non-human transgenic mammal by means of which it is possible to monitor in vivo and in all the tissues the state of activation of any intracellular receptor, utilising a reporter gene inducible by natural or synthetic molecules which modulate the activity of such receptor. The mammal in question is preferably a mouse.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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"Transgenic Mouse for Screening and for Studies of the Pharmacodynamics and Pharmacokinetics of Ligands Acting on the Oestrogen receptor and its Intracellular Receptors, and Method for the Preparation Thereof"

5

SPECIFICATION

Research carried out in the last ten years has shown that oestrogen deficiency is associated with an increased risk of the appearance of a large number of diseases of the nervous, immune, cardiovascular and bone systems. These studies thus suggest that the oestrogens, traditionally considered to be hormones responsible solely for the control of the reproductive functions, in fact have an important role in the maintenance of the homeostasis of many tissues in the mammal (for a review, see Nilsson and Gustafsson, 2000); pharmacological research is thus directed towards the obtaining of therapeutically active molecules which can be substituted for the hormone in order to exert their protective and beneficial activity solely in specific target organs.

Meanwhile, research at the molecular and cellular level has clarified the mechanism by which the hormone acts on the target cells (for a review, see Tsai and O'Malley, 1994). In particular, the hormone recognises and binds to intracellular proteins, called oestrogen receptors (so far, two types of receptor, designated by the first two letters of the Greek alphabet, are known). The hormone-receptor complex is then enabled for the recognition of specific sequences of DNA (called hormone response elements), for interaction with other protein factors (some of them ubiquitous and others tissue-specific) for the activation of the transcription of the target genes. This mechanism provides the molecular basis for the explanation of an observation made with synthetic ligands of the oestrogen receptors, namely that

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these can function as receptor agonists in some organs and antagonists in others.

At present, a considerable number of pharmaceutical multinationals are involved in the development of molecules for use in replacement
5 therapies, which may be capable of acting as activators of oestrogen receptors in some organs and as antagonists in others. For example, in the case of the oestrogen receptor, molecules are currently being sought which activate this receptor in organs such as bone and brain, but not the uterus and mammary tissue where the trophic activity of the
10 receptor could favour the onset of neoplasias.

The bottleneck in these studies comprises the identification of the molecules endowed with the desired agonistic/antagonistic activity. At present, the screening of such molecules is carried out in transformed cell lines suitably engineered to express an exogenous marker gene of
15 hormonal activity.

The limits of this type of methodology are connected with the fact that this technique is performed on immortalised/neoplastic cells, which are thus modified with respect to the physiological target; further, it does not provide any information concerning the pharmacokinetics and the
20 bioavailability of the compound with potential pharmacological activity. For these reasons, the ligands identified by screening on cells must in any case be subjected to tests on animals. Finally, not all the areas of the organism which are targets of these hormones are yet fully known. According to the present invention, it was decided to overcome these
25 limitations by the generation of a mouse engineered so that it expresses the marker gene of hormonal activity in all its cells and tissues. Hence, the administration to such a mouse of any compound with oestrogenic activity would result in an accumulation of the gene product in those organs/cells where the compound acts as a receptor agonist. This

mouse represents an ideal system for the screening of molecules acting on the oestrogen receptors in that:

1. It allows the simultaneous visualisation of all the organs/cells in which the compound under test exerts a pharmacological activity
5 (including cells whose responsivity to the hormone is unknown).
2. It makes it possible to know the distribution of the compound (for example whether it penetrates the blood-brain barrier).
3. It makes it possible also to carry out pharmacokinetic studies to clarify the catabolism of the ligand in question, and its absorption times
10 as well as its possible persistence and activity in all parts of the mouse, allowing the prediction of possible side-effects. Such methods can be carried out in vivo by non-invasive imaging methodologies.

Another use of the animal model according to the invention concerns the ecotoxicology field. One of the major environmental contamination
15 problems is connected with the activity of the so-called xenoestrogens. It has been proved that many synthetic substances widely used in agriculture (e.g. atrazine) and other compounds present in industrial wastes (e.g. dioxin) display active interference with the endocrine system in mammals causing strong repercussions on the reproductive
20 system right up to sterility; the increase in such substances in the environment is now recognised as one of the causes of increased sterility in man (Sharpe and Skakkebaek, 1993). It will thus also be possible to use the animal as a biosensor for specific environmental pollutant substances (e.g. pesticides).

25 Finally, it will be possible to use the animal as a source of cells for in vitro culturing. Primary or immortalised cultures from different tissues can be obtained by standard cell culturing protocols. Such cultures can be used in the screening of compounds with hormonal action.

The invention here described concerns the generation of a transgenic animal. As is well-known, the transgenics are animals into whose chromosome apparatus an exogenous gene has been inserted and are capable of transmitting it to their progeny. Two basic procedures exist for the generation of transgenic animals which were initially developed in the mouse: 1. the microinjection of the fragment of DNA into the genome at the single-cell stage, in the male pronucleus of a fertilised oocyte and its reimplantation into pseudogavid females (Constantini and Lacy, 1981; Gordon and Ruddle, 1981); 2. the transfection of the DNA into embryonic stem cells and the injection of these into the blastocysts (Robertson et al., 1986; Gossler et al., 1986). Various other animal species have been engineered in this way: for example Hammer and coworkers (1985) have described the application of transgenesis in rabbits, in sheep and in pigs. The mouse is still an animal much utilised in transgenesis and is also the first transgenic animal to have been patented (EP 0169672B1).

The present invention relates to a method for the production of a non-human transgenic mammalian animal by means of which it is possible to monitor in vivo and in all the tissues the activation state of an intra-cellular receptor, characterized in that a reporter gene whose transcription is inducible by synthetic or natural molecules which modulate the activity of such a receptor is incorporated into the genome of the animal.

Therefore, in the following the said reporter gene will also be referred to as reporter transgene.

More specifically, the present invention provides a method for the production of a transgenic mammalian animal utilisable as an experimental model for simultaneously monitoring in vivo the pharmacological activity, the tissue-specificity, the pharmacokinetics and the pharmaco-

dynamics of molecules with potential activity on intracellular receptors.

GENERATION OF THE ANIMAL MODEL

The transgenic model which is the object of the present invention can be obtained by the integration into the somatic and germinal line of a gene called a reporter, provided with control regions that make its
5 expression inducible by such hormones. The gene in question is not present in the genome of the mammal and codes for an enzyme easily quantifiable by an enzyme test. Any reporter gene is utilisable for the purposes of the present invention. A list of reporter genes currently
10 available are [sic] indicated, together with some of their characteristics, in Table I given later. These are luciferase, green fluorescent protein (GFP), beta-galactosidase, beta-lactamase and chloramphenicol acetyltransferase (CAT).

Also mentioned are dopamine 2 receptor (D2R) and thymidine kinase
15 (TK).

The possibility of hormone-dependently inducing the transcription of a reporter gene is a function of the presence of specific sequences to which the ligand-receptor complex binds. In particular according to the invention a series of constructs consisting of palindromic sequences
20 responsive to oestrogens (ERE) shown in Figure 2 of the attached drawings, and described later, was generated. They are:

ERE4X

ERE2X-33 bp linker-ERE2X, and

ERE2X, wherein

25 ERE2X or 4X = oestrogen-responsive element multimerised 2- or 4-fold, respectively and

33 bp linker = non-responsive DNA linking sequence between the ERE.

Of these, the construct utilised for the generation of the oestrogen-responsive transgene according to the invention preferably has the

following structure:

ERE2X.

Such sequences were placed upstream of a promoter which does not display tissue-specificity, such as the constitutive promoter of the gene
5 for viral thymidine kinase, TKprom. The promoter of the final construct is thus constituted as follows:

ERE2X-TKprom-Reporter.

A constitutive promoter according to the invention can also be a minimal promoter consisting of TATA box and transcription initiator
10 sequence.

In the tissues of the model animal presented, the administration of agonists causes an increase in the activity of the protein encoded by the reporter; this increase depends specifically on the presence of intracellular receptors activated by the ligand in the target cell and is
15 proportional to the degree of hormonal stimulation. In other words, the expression of the transgene constitutes a signal of the existence of some activity of the specific hormone in some specific body area of the transgenic mouse. The model also allows the study of molecules with antagonistic activity towards oestrogen receptors. In this case, the
20 evaluation of the antagonistic activity of the molecule in question will be effected in animals simultaneously treated with oestrogens to evaluate the blocking of the hormonal activity by the presumed antagonist.

The stable introduction of an ERE-TKprom-Reporter construct into the genome of fertilised oocytes or of embryonic stem cells makes it
25 possible to obtain a first series of individuals (called founders). From these founders are obtained, via hereditary transmission, firstly the heterozygotic individuals and then the homozygotic individuals for the transgene which will be inherited in the Mendelian manner in case of insertion into a unique site. However, the expression of the transgene
30 inserted in this manner into the animal is subject to the so-called

positional effect, namely the influence of the chromatin surrounding the site of insertion into the genome. As a result, the transgene will be expressed or not expressed, in an incorrect manner and often with undesired tissue preferences. This phenomenon, called PVE (Position
5 Variegation Effect), is typical of eukaryotic cells and has been amply characterised and studied; it was the principal cause of the failure of preceding attempts to generate the animal model which is the object of this patent. To circumvent this problem, according to the present invention suitable sequences called insulators were used, which, placed
10 at the flanks of the transgene, have the ability to isolate it from the effect of the heterochromatin and from surrounding enhancer sequences (for a review, see Fang-Lin and Elgin, 1999). Some of these sequences are shown in Table II.

The final constructs, or plasmids, utilised for the transgenesis of the
15 mouse which is the object of the present invention were constituted as follows (Figure 3):

pMAR = MAR-ERE2X-TKpr-Luciferase-MAR

pHS4 = HS4-ERE2X-TKpr-Luciferase-HS4.

The screening system developed according to the present invention
20 can be extended to any ligand acting on intracellular receptors. In this case the DNA sequence responsive to the hormone is selected among those known and available as described in Table III below, such as PRE, GRE, RARE, TRE, VD3RE and PPRE.

The Figures cited above are described below:

25 Fig.1 is the schematic representation of the starting expression plasmid containing the luciferase reporter gene under the control of the constitutive promoter of viral thymidine kinase (TK).

Fig.2 is the schematic representation of the plasmids tested for inducibility by oestrogens, in which are present the ERE sequences,
30 upstream of the TK promoter and with various arrangements: the ERE

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palindromes have been dimerised (1), or tetramerised with (3) or without (2) spacing sequences.

Fig.3 is the schematic representation of the fusion of the insulator sequences MAR and HS4 with the plasmid ERE2X-TK-luciferase.

5 Fig.4 is the schematic representation of the 0.77 Kb EcoRI/EcoRI probe used for the screening of the transgenic mice generated with the pMAR and pHS4 constructs.

PREPARATION OF THE CONSTRUCTS

The starting construct pTKluc described in Fig.1 was obtained by
10 subcloning the luciferase gene from pGL2basic (Promega) into the BamHI site of pBluescript (Stratagene); the -109 bp fragment of the TK promoter was isolated from the vector pBLCAT2 (Luckov and Schutz, 1987) and subcloned in the HindIII site of pBluescript. The construct obtained (pTKluc) was sequenced and the expression of luciferase was
15 verified in MCF-7 human mammary carcinoma cells.

The oestrogen-responsive sequence (ERE), see Table III below, multimerised in the various arrangements described in Figure 2, was subcloned in the Sall site of pTKluc; the oestrogen-responsivity of the constructs thus obtained was examined in MCF-7 cells and in human
20 neuroblastoma cells SK-N-BE. The highest responsivity to oestradiol was obtained from the construct pERE2X-TKluc (Figure 2) which was chosen for generation of the final vectors utilised in the transgenesis.

The insulators HS4 (Chung et al., 1993) and MAR (Stief et al., 1989) were subcloned into the flanking regions of pERE2XTKluc in the KpnI
25 and NotI sites generating the vectors pHS4 and pMAR (Figure 3).

The probe used in the screening of the transgenic animals (Figure 4) was obtained by digestion of the plasmid pERE2XTKluc with the restriction enzyme EcoRI and the 0.77 Kb fragment was separated by electrophoresis on agarose gel and purified by electroelution (Maniatis
30 et al., 1982).

PRODUCTION OF TRANSGENIC MICE

The DNA fragments containing the transgenes were obtained from the plasmids pMAR and pHS4 by digesting them with the restriction enzyme BssHII and thus releasing the inserts of 8.95 Kb and 7.85 Kb respectively (Figure 3); the transgenes were purified by electrophoresis on agarose gel and subsequent electroelution procedure (Maniatis et al., 1982). About 400 copies of the insert thus purified were injected into the male pronucleus of a fertilised egg deriving from individuals of the B6D2F1 strain of mouse. The mice were obtained from the Charles River Laboratories. The injected eggs were reimplanted into pseudo-gravid females. In this way, about 100 individuals were obtained. At the age of four weeks, the DNA from the biopsy of the tails of these individuals was extracted (Maniatis et al., 1982). About 10 µg of DNA for each sample were immobilised with a Slot Blot device (Schleicher and Schuell) on nylon filters (HybondN, Amersham). The filters obtained were subjected to hybridisation experiments with the 0.7 Kb EcoRI/EcoRI DNA probe described in Fig.4, labelled with ³²P using a kit based on the multiprimer principle, according to the producer's instructions (Megaprime DNA labelling system, Amersham). The hybridisation procedure was effected essentially as previously described (Maniatis et al., 1982); in particular, the hybridisation temperature used was 60°C and the washings were performed in a solution containing 0.1 x SSC/ 0.1% SDS (3 washings of 10 minutes at ambient temperature and 2 washings of 30 minutes at 60°C).

The screening of the founders, effected as previously described, made it possible to identify 17 mice with the transgene integrated: 10 pMAR and 7 pHS4. Only 12 founders succeeded in generating fertile progeny positive for the presence of the transgene.

The number of copies integrated in the different lines is variable from one to thirty.

The founders were crossed with non-injected animals from the same original strain (B6D2F1) and the new-born at the age of four weeks
5 were analysed for the presence of the transgene as described for the founders, by means of the Slot Blot technique.

Through successive recrossing of each of the 12 fertile lines, heterozygotic individuals and in the end also homozygotic lines were obtained.
ANALYSIS OF THE TRANSGENIC MICE

10 To verify the expression of the transgene in the different tissues and its inducibility by oestrogens, 4 independent experiments were carried out on 8 female individuals of each line (4 heterozygotic and 4 homozygotic). For each experiment, two female individuals were ovariectomised. With the aim of reducing the oestrogens present in the blood-
15 stream of the ovariectomised animals below the detection limit, there was a wait of two weeks before the induction experiment was carried out; the individuals were then subjected to subcutaneous (s.c.) injection of mineral oil with or without 50 µg/kg of dissolved 17-β oestradiol; 16 hours after the treatment, the individuals were sacrificed and the
20 following tissues were dissected out of them: uterus, liver, spleen, lung, heart, kidney, hypothalamus and brain. The protein extracts were obtained by disintegration of the tissue with a homogenizer in the presence of a phosphate lysis buffer consisting of: 0.1 M KH₂PO₄, 0.1 M K₂HPO₄, 1 mM DTT, 4 mM EGTA and 4 mM EDTA at pH 7.8. The
25 suspension was frozen and thawed three times, and centrifuged for 30 minutes at 13,000 g; the supernatant was collected and equal quantities of protein, measured by the colorimetric method of Bradford, were subjected to enzymatic testing to reveal the activity of the enzyme luciferase. The method for detection of the activity of the reporter was
30 carried out with a kit by the procedure recommended by the producer

(Sigma), mixing ca. 20 µg of protein extract with the luciferin substrate in the presence of 0.5 mM ATP and 30 mM DTT and measuring the fluorescence emitted using a luminometer (Lumat BL 9500, Berthold). With reference to Figures 5 to 7 of the attached drawings, individuals
5 were subcutaneously injected (s.c.) with 50 µg/Kg 17β-estradiol (E2) or with vehicle (vegetable oil) and sacrificed at 24 hours. Luciferase enzymatic activity was evaluated and plotted in the graph. The arbitrary units are obtained considering the luciferase activity in the uterus of control individuals =100.

10 Fig.5 shows the expression of luciferase in line 2 of the transgenic mice treated with 50 µg/kg of 17-β oestradiol (s.c.) for 24 hours, Figure 6 shows the expression of luciferase in line 61 of the transgenic mice treated with 50 µg/kg of 17-β oestradiol (s.c.) for 24 hours, and Figure 7 shows the expression of luciferase in line 59 of the transgenic mice
15 treated with 50 µg/kg of 17-β oestradiol (s.c.) for 24 hours.

Of the 10 lines analysed, one (line 2, transgene pMAR) displayed ubiquitous and inducible expression of the transgene (Fig.5); one was found to be inducible predominantly in the brain and in the liver (line 61, transgene pHS4) (Fig.6), and one is inducible in the brain and in the
20 hypothalamus (line 59, transgene pMAR) (Fig.7).

TEST FOR THE AGONISTIC AND ANTAGONISTIC ACTION OF A COMPOUND AND FOR THE ACTIVITY OF XENOOESTROGENS

The animal of this invention can be utilised to test the ability of a compound to behave as an agonist or as an antagonist in the various
25 tissues. By subcutaneous injection of the substance in the presence or in the absence of 17-β oestradiol it is in fact possible to compare the distribution and the intensity of expression of the luciferase gene in the various tissues and to compare it with the control or with the injection of 17-β oestradiol alone. The effect of in vivo administration of two known
30 ER antagonists was investigated in line 2 of transgenic mice. Figure 8

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(upper panel) shows blockade of 17- β oestradiol (E2) activation by 4-hydroxytamoxifen (T) and ICI 182,780 (ICI) and partial agonist activity of tamoxifen (T) in liver and brain. Bars represent the average \pm s.e.m of 5-7 mice. *($P < 0.01$ as compared to the control), °($P < 0.01$ as compared to the E2-treated); P were calculated with ANOVA followed by Scheffé test. Ligands were administered at the following doses: E2 50 μ g/Kg, 4-hydroxytamoxifen and ICI 182,780 250 μ g/Kg. Luciferase is expressed as fold induction obtained as the ratio between luciferase activity in ligands treated/control mice.

10 With reference to the upper panel of Figure 8, s.c. administration of 250 μ g/kg of 4-hydroxy-tamoxifen for 6 hours increased the level of luciferase in the liver 12-fold and 7-fold respectively, confirming in vivo the partial agonist activity of 4-hydroxytamoxifen in these tissues. The injection of 250 μ g/kg of tamoxifen or ICI 182,780 one hour before the
15 administration of 50 μ g/kg of E2 inhibits the E2-dependent activation of luciferase expression as expected from the antagonistic effect of the compounds with respect to E2.

The animal of this invention can be utilised to verify the interference of environmental contaminants (pesticides, fertilisers, etc.) with the
20 endocrine system, by administration of these by the transdermal or sub-cutaneous route or via the diet. The effects of a paradigmatic xeno-oestrogen, the organochlorine DDT, were investigated in our model. Figure 8 (lower panel) shows the luciferase activity evaluated in the liver and brain (two known target organs for DDT toxicity) of individuals
25 injected s.c. with 5000 μ g/ml DDT and sacrificed at 3, 6, 16, 24, 48 or 72 hours. As shown, at 24 hours, maximal induction of luciferase activity was detected in both liver and brain compared to a control animal injected with vegetable oil (vehicle); this induction persisted until 72 hours.

PHARMACOKINETICS AND PHARMACODYNAMICS OF E2 IN THE TRANSGENIC ANIMALS

The pharmacokinetics of any compound acting via intracellular receptors can be studied in the animal of the invention, if the reporter
5 used codes for a protein with a fast turnover in mammalian cells (e.g. luciferase, which has a 3 hour half-life in mammalian cells). This feature is required in order to follow the variation in receptor activation with time. Figure 9 (upper panel) shows a pharmacokinetic study of E2 in the transgenic animal generated; individuals were injected s.c. with
10 50 µg/kg of E2 and sacrificed after 3, 6 or 16 hours. The maximal luciferase accumulation was observed at 6 hours in bone and liver, which correlates with the expected peak of oestrogen receptor transcriptional activity. Luciferase activity is expressed as fold induction as defined in Fig.8. Bars are representative of the average values of at
15 least 5 individuals.

The pharmacodynamics of any compound acting via intracellular receptors can also be correctly predicted by the transgenic model of the invention. As an illustrative example, ovariectomised luciferase activity was measured in 26 different tissues from 2-month old female mice
20 which had been ovariectomised 2 weeks prior to the experiment. Mice were treated for 16 hours with either vehicle or E2 subcutaneously (s.c.). Fig.9 (lower panel) shows that hormonal treatment induced an increase in the enzyme content compared to controls in oestrogen target organs. This distribution is very consistent with the reported
25 tissue distribution of oestrogen receptors.

TISSUE CULTURES

The tissues of the animal which is the object of this invention can further be utilised as sources of cells for in vitro culture by means of standard culturing techniques. These techniques make it possible to
30 obtain primary cultures which can be utilised directly as nontransformed

lines for the screening of substances with oestrogenic activity, or can be transformed in order to obtain lines whose cells continue to proliferate. As an example of such an application, bone marrow cells from the transgenic mouse generated were obtained and used in a pharmacol-
5 ological study; the results are summarised in Figure 10. Two millions of bone marrow cells were suspended in phenol red-free RPMI 1640 with 10% stripped serum. Cells were treated with vehicle (C), with increasing concentrations of E2 (0,001; 0,01; 0,1; 1 and 10 nM), with 100 nM ICI 182,780 (ICI) with 4-hydroxytamoxifen (T) either alone or with 1 nM E2;
10 progesterone (Prog) and dexametasone (Dex) were used at 10 nM final concentration. Bars represent the average \pm s.e.m. of 5 individual experiments each done in triplicate. *(P<0.01 as compared to the control), **(P<0.005 as compared to the control), °(P<0.05 as compared to the T-treated); P were calculated with ANOVA followed by Scheffé
15 test. The cells were treated for 16 hours with increasing concentrations of E2 (0.01-10 nM) or with 100 nM of two ER antagonists: 4-hydroxytamoxifen (T) and ICI 182,780 (ICI) alone or in the presence of 1 nM E2. E2 induced a dose-dependent increase in luciferase accumulation blocked by the presence of ICI 182,780. ICI 182,780 by
20 itself did not sort [sic] any effect. Conversely, 4-hydroxytamoxifen induced a significant increase in luciferase levels even though lower than E2 at the same concentration. On co-administration with E2, 4-hydroxytamoxifen induced higher luciferase accumulation, yet the level reached was still lower than with E2 alone. This is compatible with the
25 partial agonist activity of 4-hydroxytamoxifen and with the fact that it is present in the solution at a concentration 100 times higher than that of E2. As a control, we also tested progesterone and dexamethasone (10 nM). Both ligands did not have any effect on the ER reporter. Taken
30 together, these data confirm that primary cells can be explanted from the engineered mice; the transgene is controlled by ligands of ER with

modalities recapitulating those reported for the natural target genes.

DEPOSITS

The plasmids utilised for the transgenesis, described in Figure 3, have been deposited at the ECACC (European Collection of Cell
5 Cultures) Institute, Salisbury, Wiltshire SP4 0JG, UK and have been given the following access numbers: pMAR00041120 and pHS400041121.

The sequences of the plasmids have been deposited at the EMBL (European Molecular Biology Laboratory) Institute, Heidelberg,
10 Germany, and have been given the following access numbers: pHS4 = AJ277959 and pMAR = A277960.

Finally, the list of literature references cited is given.

- Costantini F and Lacy E., (1981) *Nature*, 294: 92-94.
- Chalfie M., Tu Y., Euskirchen G., Ward W. W. and Prasher D. C. (1994)
15 *Science*, 263: 802-805.
- Chung J.H., Whiteley M and Felsenfeld G. (1993) *Cell*, 74: 505-514.
- De The H., Vivanco Ruiz M.D.M., Tiollais P., Stunnenberg H. and Dejean A. (1990) *Nature*, 343, 177-180.
- de Wet J.R. et al. (1987) *Mol. Cell. Biology*, 7: 725.
- 20 Durand B., Saunders M., Leroy P., Leid M. and Chambon P. (1992) *Cell*, 71: 73-86.
- Fang-Lin S. and Elgin S.C.R. (1999) *Cell*, 99: 459-462.
- Fu Y., Wang Y and Evans S.M. (1998) *Nature Biotechnology*, 16: 253-257.
- 25 Gordon J.W. and Ruddle F. H. (1981) *Science*, 214: 1244-1246.
- Gorman C.M., Moffat L.F. and Howard B.H. (1982) *Mol. Cell. Biol.*, 2: 1044.
- Gossler A.T., Doetschman R., Korn E. and Kemler R. (1986) *Proc. Natl. Acad. Sci. USA*, 83: 9065-9069.
- 30 Klein-Hitpass L., Schorpp M., Wagner U., Ryffel G.U., Klierer S.A. and

- Umesono K. (1986) *Cell*, 46: 1053-61.
- Noonan D.J., Heyman R.A. and Evans R.M. (1992) *Nature*, 358: 771-774.
- Luckow B. and Schutz G. (1987) *Nucleic Acids Res.*, 15: 5490.
- 5 Maniatis T., Fritsch E.F. and Sambrook J. (1982) Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.
- Miller J.H. (1972) Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.
- Nilsson S and Gustafsson J-A (2000) in press.
- 10 Noda M., Vogel R., Craig A.M., Prah J., DeLuca H.F. and Denhardt D.T. (1990) *Proc. Natl. Acad. Sci. USA*, 87: 9995-9999.
- Robertson E.A., Bradley M. and Evans M. (1986) *Nature*, 323: 445-448.
- Sap J., de Magistris L., Scmitt J., Stunnenberg H. and Vennestrom B. (1990) *EMBO J.*, 9: 887-896.
- 15 Sharpe R.M. and Skakkebaek N.E. (1993) *Lancet*, 341: 1392-1395.
- Stief A., Winter D., Stratling W.H. and Stippel A.E. (1989) *Nature*, 341: 343-345.
- Stunnenberg H.G. (1993) *Bioessays*, 15: 309-15.
- Tsai M-J. and O'Malley B.W. (1994) *Ann. Rev. Biochem.*, 63: 451-486.
- 20 von der Ahe D., Janich S., Scheidereit C., Renkawitz R., Schutz G. and Beato M. (1985) *Nature*, 313: 706-709.
- Zlokarnik G., Negulescu P.A., Knapp T.E., Mere L., Burres N., Feng L., Whitney M., Roemer K. and Tsien R.Y. (1998) *Science*, 279: 84-88.

Table I

| Reporter | Enzymatic test | Detection in vital cells | References |
|------------------------------------|----------------|--------------------------|------------------------|
| Luciferase | yes | yes | de Wet 1987 |
| Green fluorescent protein | no | yes | Chalfie et al., 1994 |
| beta galactosidase | yes | no | Miller 1982 |
| beta lactamase | yes | yes | Zlokarnik et al., 1998 |
| chloramphenicol acetyl-transferase | yes | no | Gorman et al., 1982 |

Table II

| Insulator | Gene of origin | References |
|-----------|------------------------|--------------------|
| HS4 | human beta-globin | Chung et al., 1993 |
| MAR | chicken lysozyme | Stief et al., 1989 |
| ITR | adeno-associated virus | Fu et al., 1998 |

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Table III

| Responsive sequence | Sequence recognised | Gene regulated by the responsive element cited | Receptor that binds the responsive sequence | References |
|---------------------|---|--|---|---|
| ERE | ggtca(n) ₃ tgacc | vitellogenin | oestrogen receptor | Klein-Hitpass L, et al., 1986 |
| GRE/PRE | agaaca(n) ₃ gtgtct | MMTV | glucocorticoid and progesterone receptors | von der Ahe D, et al., 1985 |
| RARE | ggttca(n) ₅ agttca agttca(n) ₂ aggcca agttcanaggtca | RAR- β 2 CRABP II CRABP II | retinoic acid receptor | De The et al, 1990 Durand et al., 1992 |
| TRE | gggtca(n) ₄ aggtcc | growth hormone | thyroid hormone receptor | Sap et al., 1990 |
| VD ₃ RE | gttca(n) ₃ ggttca | osteopontin | vitamin D receptor | Noda et al, 1990 |
| PPRE | aggtcanaaggtca | acyl CoA oxidase | peroxysome proliferation factor receptor | Kliwer et al, 1992 |

CLAIMS

1. A method for the production of a non-human transgenic mammalian animal by means of which it is possible to monitor in vivo and in all the tissues the state of activation of an intracellular receptor,
5 characterized in that a reporter gene, the transcription of which is inducible by natural or synthetic molecules which modulate the activity of such receptor, is incorporated into the genome of the said animal.
2. A method according to Claim 1, characterized in that the said reporter transgene is ligated to insulator sequences, sequences
10 responsive to intracellular hormones, and constitutive promoters.
3. A method according to Claim 1, characterized in that the said reporter transgene is selected among: luciferase, green fluorescent protein (GFP), beta-galactosidase, beta-lactamase, chloramphenicol acetyltransferase (CAT), dopamine 2 receptor (D2R) and thymidine
15 kinase (TK).
4. A method according to Claims 1 and 2, characterized in that the induction of the said transcription by the said molecules is governed by the insertion into the said transgene of one of the following sequences responsive to the said molecules which modulate the activity
20 of such receptor: ERE, PRE, GRE, RERE, TRE, VD3RE or PPRE.
5. A method according to Claims 2 and 4, characterized in that one of the following constitutive promoters is ligated to the said sequences: viral thymidine kinase promoter (TK) or minimum promoter consisting of TATA box and transcription-initiator sequence.
- 25 6. Method according to Claim 5, characterized in that the resulting construct is constituted as follows:
ERE2X-TKprom-Reporter.
7. A method according to Claims 2 and 4, characterized in that the insulators MAR, HS4 or ITR are ligated to the said sequences by

subcloning them into the regions flanking the said transgene, so giving rise to a plasmid.

8. Method according to Claim 7, characterized in that the said plasmids are constituted as follows:

5 pMAR = MAR-ERE2X-TKpr-Luciferase-MAR

PHA4 = HS4-ERE2X-TKpr-Luciferase-HS4.

9. A method according to Claim 8, characterized in that sequences of the said plasmids defined by the EMBL access numbers pHS4 = AJ277959 and pMAR = AJ277960 are used.

10 10. A method according to Claim 1, which provides for the incorporation of the said transgene into the genome at a stage not higher than the single embryonic cell.

11. A method according to Claim 1, which provides for the incorporation of the said transgene into the genome at a stage not
15 higher than 8 embryonic cells.

12. A method according to Claim 1, characterized in that it provides for the injection of the said transgene into the male pronucleus of a fertilised egg of the said animal.

13. A method for checking the agonistic and antagonistic action
20 of ligands for intracellular receptors, characterized in that a transgenic animal generated with one of the methods stated in the foregoing claims is used.

14. A method for studying the pharmacokinetics and the pharmacokinetics combined to the pharmacodynamics of any
25 compound acting through intracellular receptors in order to evaluate the biotransformation or the dynamics of compound absorption, distribution and elimination within the tissues of the said animal, characterized in that a transgenic animal as generated by a method according to one or more of the foregoing claims is used.

15. A method for performing external monitoring of compounds in living mammals by non-invasive imaging, characterized in that a transgenic animal as generated by a method according to one or more of the foregoing claims is used.

5 16. A method for checking the action of pesticides, insecticides or in general of environmental contaminants, on the endocrine system, characterized in that a transgenic animal generated by one of the methods stated in the foregoing claims is utilised.

10 17. Plasmids defined by the ECACC access number pMAR00041120 and pHS400041121.

18. A sequence of plasmids defined by the EMBL access number pHS4 = AJ277959 and pMAR = AJ277960.

19. A chromosome of a transgenic animal generated by the method of the foregoing claims.

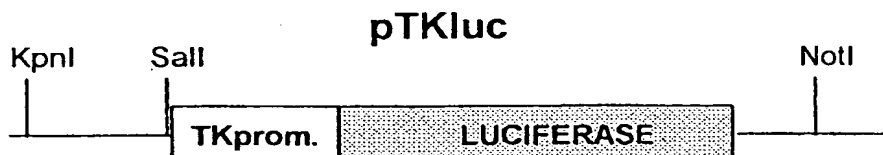
15 20. A method for providing cells in culture deriving from a transgenic animal as defined in any one of the foregoing claims.

21. A cell derived from the soma obtained from a transgenic animal as defined in any one of the foregoing claims.

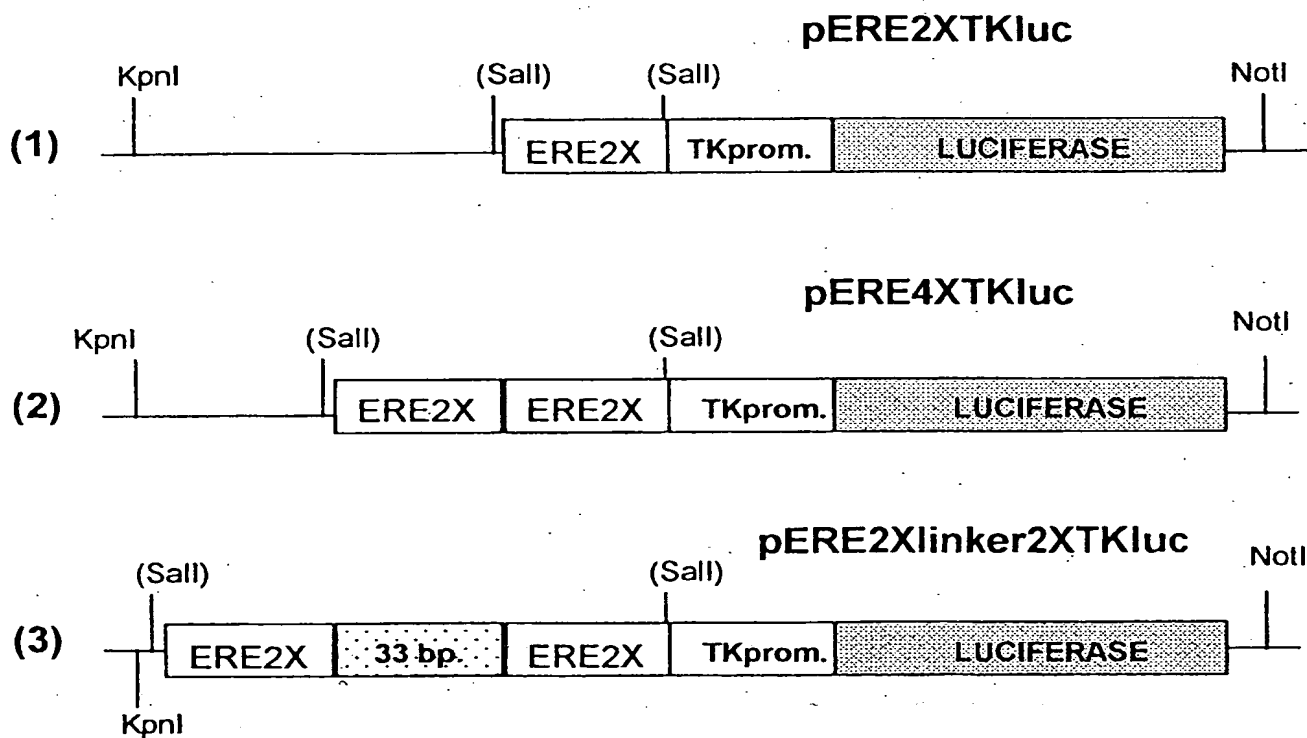
20 22. Non-human transgenic mammal with genome modified according to the method of any one of the foregoing claims.

23. Transgenic mammal according to Claim 22, characterized in that it is a mouse.



**FIG. 1**

Starting construct containing the TK (-109) promoter.

**FIG. 2**

Ligation of the multimerized ERE sequences upstream the promoter region.

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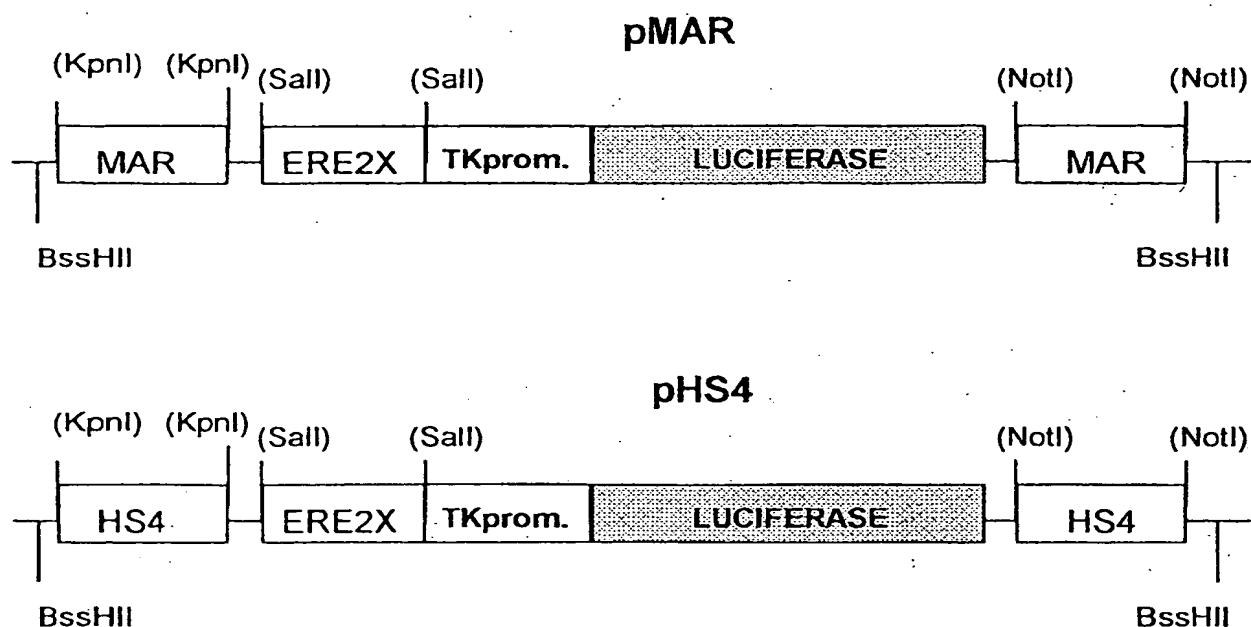


FIG. 3

Final constructs - Ligation of the MAR and HS4 sequences in the flanking regions of the pERE2XTKluc construct.

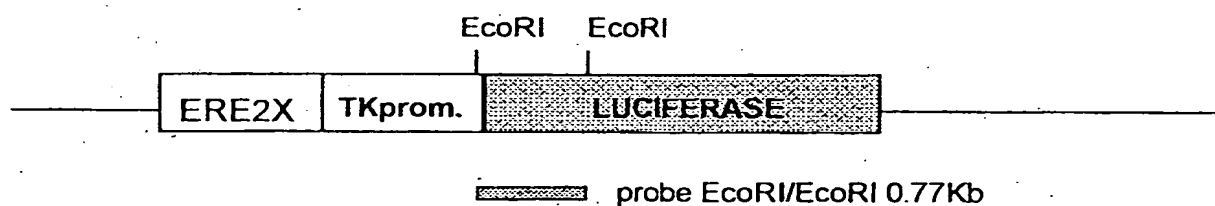


FIG. 4

EcoRI/EcoRI 0.77 Kb probe used for the screening of transgenic mice generated with pMAR and pHs4 constructs.

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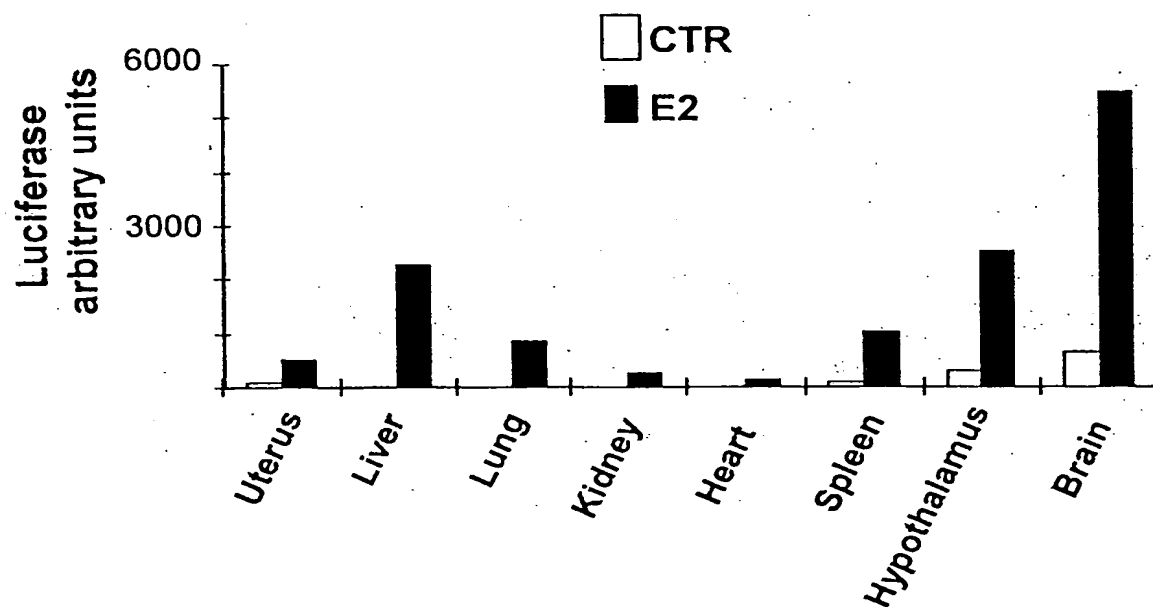


FIG. 5
Luciferase activity in line 2 of transgenic mice.

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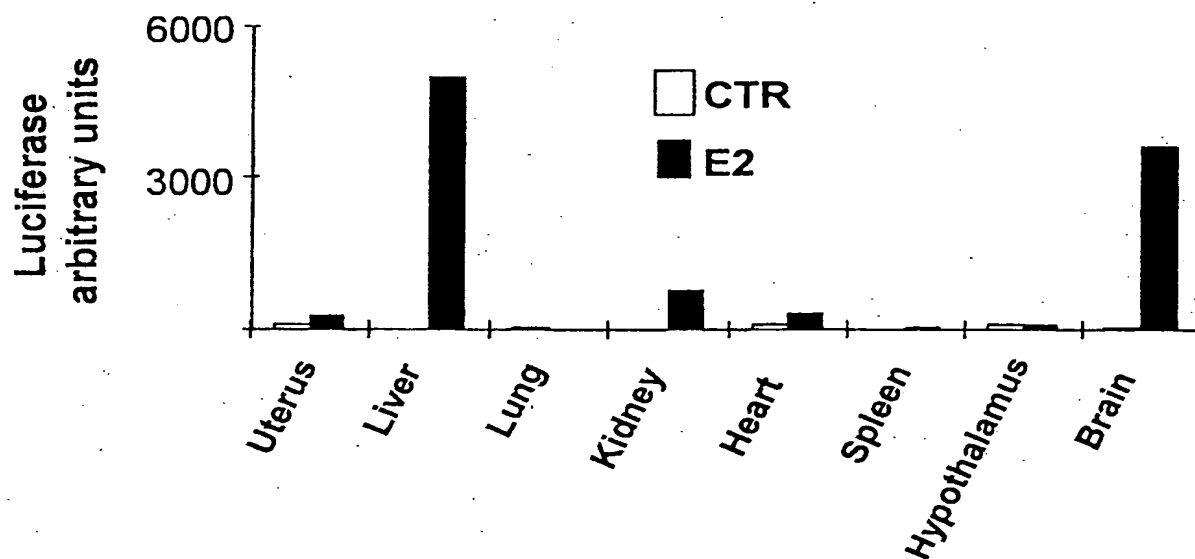


FIG. 6
Luciferase activity in line 61.

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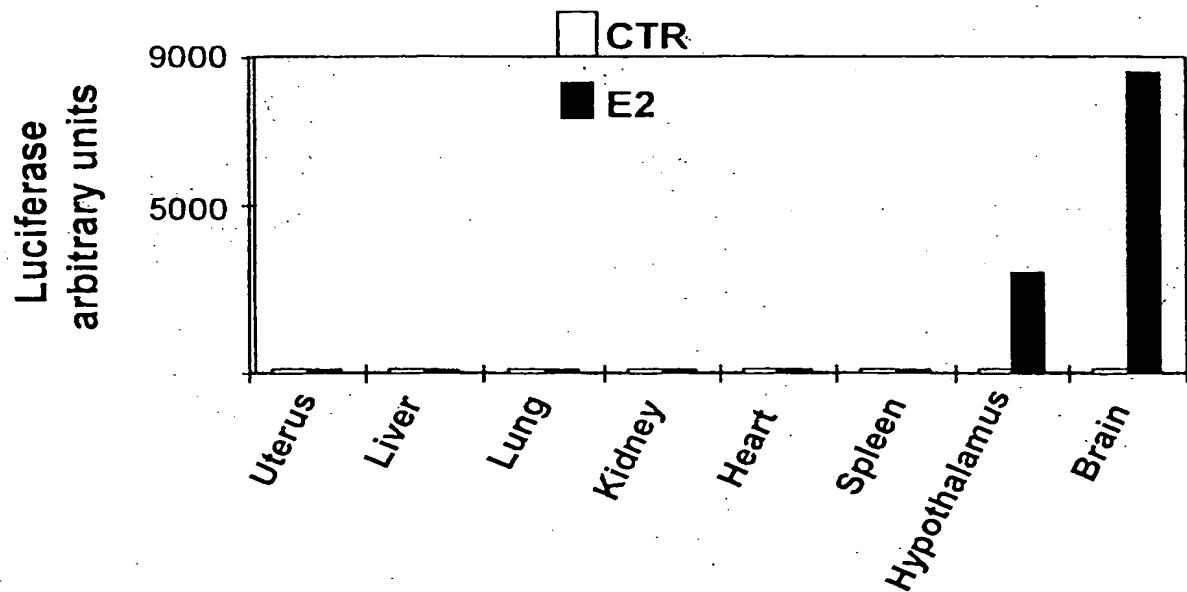


FIG. 7
Luciferase activity in line 59 of transgenic mice.

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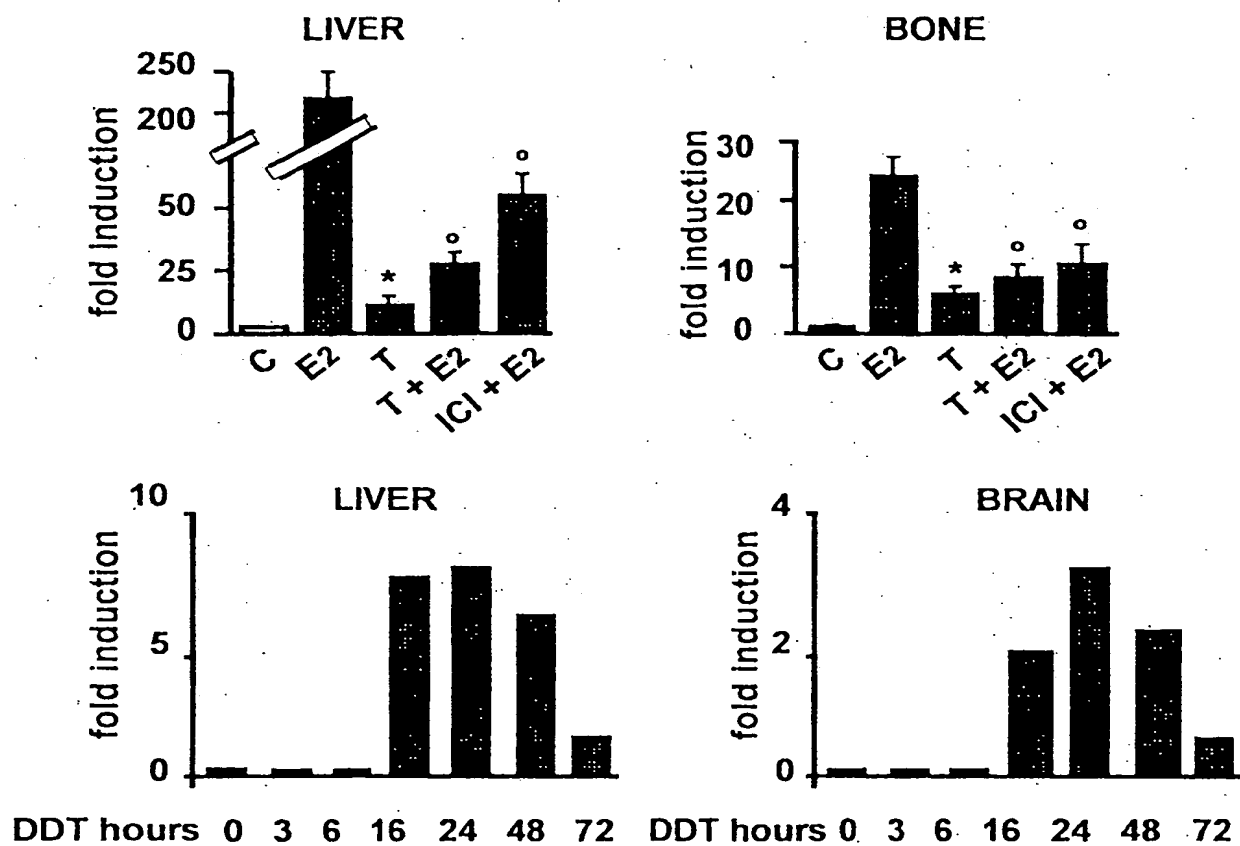
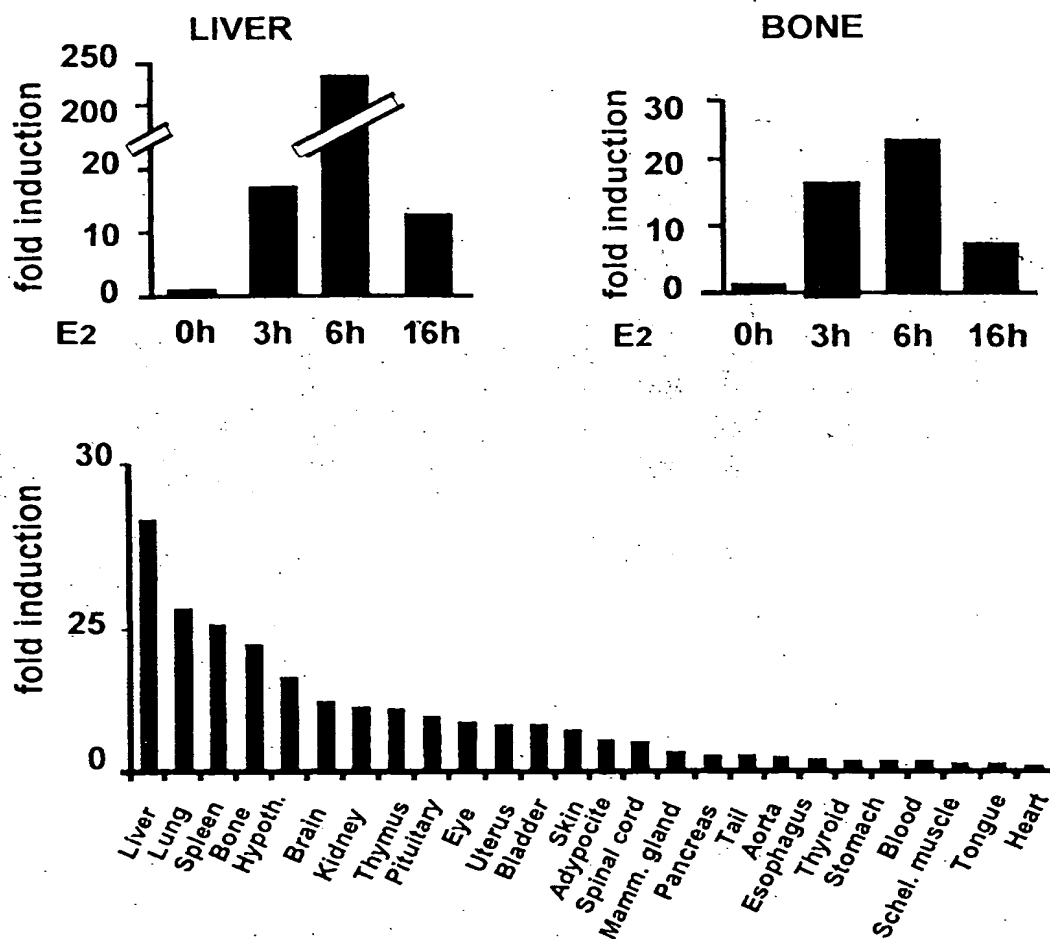


FIG. 8

Pharmacological modulation of luciferase activity *in vivo*.
Luciferase activity in bone and liver protein extracts of two
months old male mice.

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**FIG. 9**

Upper panel: time course of luciferase activity in liver and bone of transgenic mice treated with 17β-estradiol (E2). Lower panel: tissue distribution of the E2-dependent luciferase induction within several tissues.

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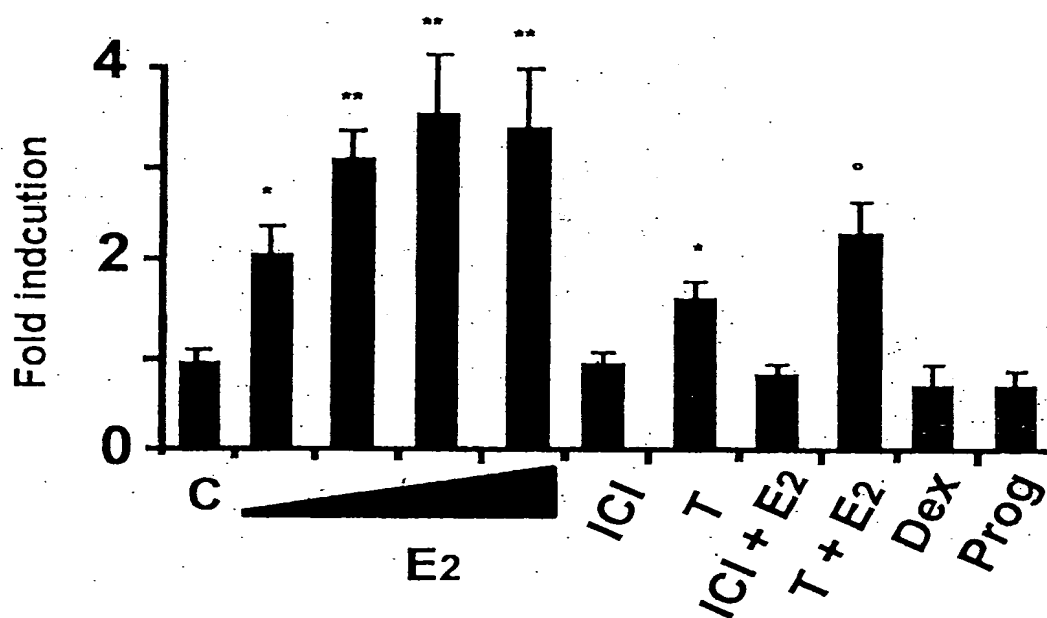


FIG. 10

Expression of luciferase in primary bone marrow cells from transgenic mice.

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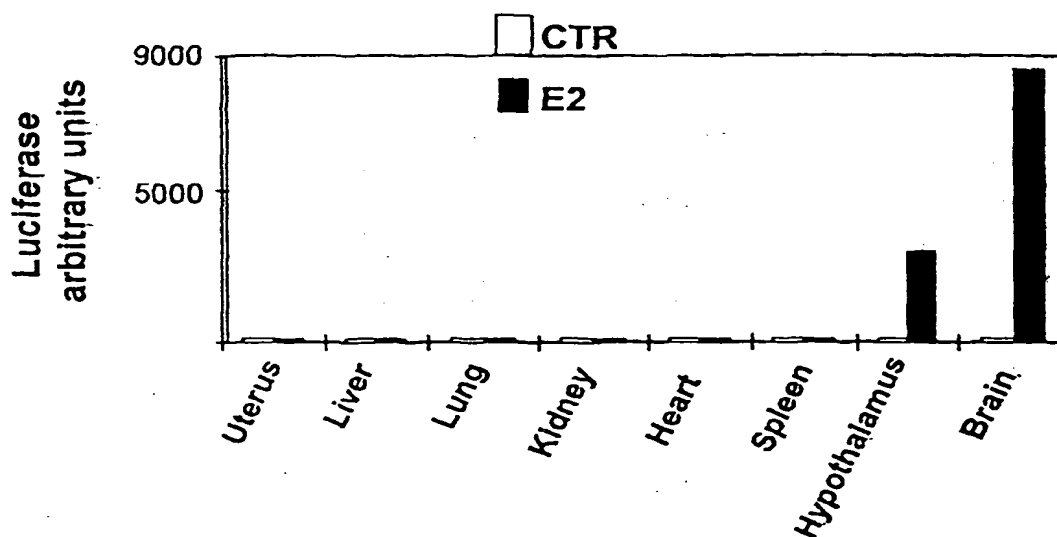
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(54) Title: TRANSGENIC MOUSE FOR SCREENING AND FOR STUDIES OF THE PHARMACODYNAMICS AND PHAR-
MACOKINETICS OF LIGANDS ACTING ON THE OESTROGEN RECEPTOR AND ITS INTRACELLULAR RECEPTORS,
AND METHOD FOR THE PREPARATION THEREOF



Luciferase activity in line 59 of transgenic mice.

(57) Abstract: The object of the invention is a method for the production of a non-human transgenic mammal by means of which it is possible to monitor in vivo and in all the tissues the state of activation of any intracellular receptor, utilising a reporter gene inducible by natural or synthetic molecules which modulate the activity of such receptor. The mammal in question is preferably a mouse.

WO 02/01949 A3



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C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------|
| X | KARPERIEN MARCEL ET AL: "Identification of a retinoic acid-inducible element in the murine PTH/PTHrP (parathyroid hormone/parathyroid hormone-related peptide) receptor gene." MOLECULAR ENDOCRINOLOGY, vol. 13, no. 7, July 1999 (1999-07), pages 1183-1196, XP001026840 ISSN: 0888-8809 | 1,3,5, 10-16, 19-23 |
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| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|----------|--|---------------------------|
| X | SOLOMIN L ET AL: "Retinoid-X receptor signalling in the developing spinal cord" NATURE, MACMILLAN JOURNALS LTD. LONDON, GB, vol. 395, 24 September 1998 (1998-09-24), pages 398-402, XP002134853 ISSN: 0028-0836 the whole document | 1,3, 10-16, 19-23 |
| Y | --- | 2,4-9, 17,18 |
| X | WO 96 40911 A (BAYLOR COLLEGE MEDICINE) 19 December 1996 (1996-12-19) page 15, line 31 -page 16, line 4 page 36, line 11 -page 37, line 3 page 61, line 10 -page 61, line 34 | 1,3-5, 10-16, 19-23 |
| Y | --- | 2,6-9, 17,18 |
| Y | VANDERKUIJER J A ET AL: "Effects of estradiol-17-beta analogues on activation of estrogen response element regulated chloramphenicol acetyltransferase expression." BIOCHEMISTRY, vol. 32, no. 27, 1993, pages 7016-7021, XP001026876 ISSN: 0006-2960 the whole document | 1-23 |
| Y | ARNOLD STEVEN F ET AL: "A yeast estrogen screen for examining the relative exposure of cells to natural and xenoestrogens." ENVIRONMENTAL HEALTH PERSPECTIVES, vol. 104, no. 5, 1996, pages 544-548, XP001026841 ISSN: 0091-6765 the whole document | 1-23 |
| Y | KLEIN-HITPASS L ET AL: "SYNERGISM OF CLOSELY ADJACENT ESTROGEN-RESPONSIVE ELEMENTS INCREASES THEIR REGULATORY POTENTIAL" JOURNAL OF MOLECULAR BIOLOGY, vol. 201, no. 3, 1988, pages 537-544, XP001026878 ISSN: 0022-2836 the whole document | 6,8,9, 17,18 |
| | --- | |
| | -/-- | |

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| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|----------|--|-----------------------|
| Y | ALLEN GEORGE C ET AL: "Use of matrix attachment regions (MARs) to minimize transgene silencing." PLANT MOLECULAR BIOLOGY, vol. 43, no. 2-3, June 2000 (2000-06), pages 361-376, XP001038358 ISSN: 0167-4412 the whole document | 2,7-9, 17,18 |
| Y | SUN FANG-LIN ET AL: "Putting boundaries on silence." CELL, vol. 99, no. 5, 24 November 1999 (1999-11-24), pages 459-462, XP002190337 ISSN: 0092-8674 the whole document | 2,7-9, 17,18 |
| Y | MIGLIACCIO S ET AL: "ESTROGENS MODULATE THE RESPONSIVENESS OF OSTEOBLAST-LIKE CELLS ROS 17-2.8 STABLY TRANSFECTED WITH ESTROGEN RECEPTOR" ENDOCRINOLOGY, vol. 130, no. 5, 1992, pages 2617-2624, XP001026842 ISSN: 0013-7227 the whole document | 1-23 |

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 01/07622

| Patent document cited in search report | | Publication date | Patent family member(s) | Publication date |
|---|---|---------------------|----------------------------|---------------------|
| WO 9640911 | A | 19-12-1996 | AU 5378196 A | 30-12-1996 |
| | | | CA 2223717 A1 | 19-12-1996 |
| | | | EP 0832217 A1 | 01-04-1998 |
| | | | JP 11514842 T | 21-12-1999 |
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